BACTERIAL CYTOCHROMES

LUCILE SMITH

Johnson Foundation for Medical Physics, University of Pennsylvania, Philadelphia, Pennsylvania

I. INTRODUCTION

In his first paper on the cytochrome system in 1925, Keilin (1) noted that the cytochromes have a widespread distribution in nature. These pigments are characterized by sharp bands in the absorption spectrum when they are in the reduced form, and the appearance and disappearance of these bands could be seen with a visual spectroscope as the cytochromes were reduced and oxidized in various kinds of cells of animals and higher plants and in bacteria, except for the obligate anaerobes.

The reactions of the cytochrome system have now been studied extensively. Many studies have been made with baker's yeast and with a preparation of heart muscle extract devised by Keilin and Hartree (2). The heart muscle extract has a high concentration of cytochromes and less turbidity than suspensions of whole cells or homogenates; thus, it is more suitable for spectroscopic observations. The cytochrome systems of yeast and heart muscle are found to be similar. The α-bands of the reduced pigments in the visible region of the spectrum can be seen at 605, 564 and 551 mμ, corresponding to a mixture of cytochromes a + a_3, b and c, respectively. Like other hemoproteins, these cytochromes also show secondary or β-bands in the visible region, which overlap to form one inhomogeneous band around 525 mμ. The γ-bands (often called Soret bands) of these cytochromes are strong bands in the violet region of the spectrum at 445 mμ (cytochromes a + a_3), 430 mμ (cytochrome b) and 415 mμ (cytochrome c) in the reduced form. The Soret bands are very difficult to see with a visual spectroscope. It was Keilin and Hartree (3) who showed that the bands at 605 and 445 mμ in heart muscle extract correspond to a mixture of two cytochromes, a and a_3, and that the large band at 445 mμ is mostly due to cytochrome a_3, while cytochrome a is mostly responsible for the visible band at 605 mμ. They observed that cytochrome a_3 has a number of properties of the enzyme cytochrome c oxidase and that the absorption bands of its carbon monoxide compound correspond to those observed by Kubowitz and Haas (4) for their "respiratory enzyme".

The cytochrome a_3 of baker's yeast and heart muscle has now been definitely identified as the respiratory enzyme of these cells (5), which is cytochrome c oxidase. The cytochrome a_3 oxidase reacts with oxygen; it oxidizes cytochrome c which can oxidize a number of other substances in the cell. Cytochrome c has been isolated from the cells and purified (6); thus its properties have been studied extensively. Definite roles for cytochromes a and b have not been established. However, they can also be seen to undergo oxidation and reduction in the intact cells.

One more cytochrome has been observed by Keilin and Hartree in yeast and heart muscle (7). The absorption band of this cytochrome, which they called cytochrome e, can be seen only on cooling the cells in liquid air. This procedure intensifies and sharpens the absorption bands of the cytochromes.

A recent article by Keilin and Slater (8) reviews the developments leading up to our present concepts of the mammalian cytochrome system, and Wyman's review of the hemoproteins (9) gives an insight into the structure and properties of these pigments. In an attempt to give some sort of over-all picture of the nature of these pigments, this review will compare and contrast the bacterial cytochrome systems with that of mammalian tissues, which has been investigated more thoroughly.

II. OBSERVATIONS ON THE ABSORPTION SPECTRA OF BACTERIAL CYTOCHROMES

A. Studies with the Visual Spectroscope

Several years after Keilin's first description of the cytochromes, Keilin (10) and Yaoi and Tamiya (11) reported that the bands of the reduced cytochromes of many bacteria were not at the same wavelengths as those of yeast and mammalian tissues. Yaoi and Tamiya concluded from their studies that the aerobic bacteria showed a 4-banded cytochrome spectrum, the facultative aerobes a 2- or 3-banded spectrum,

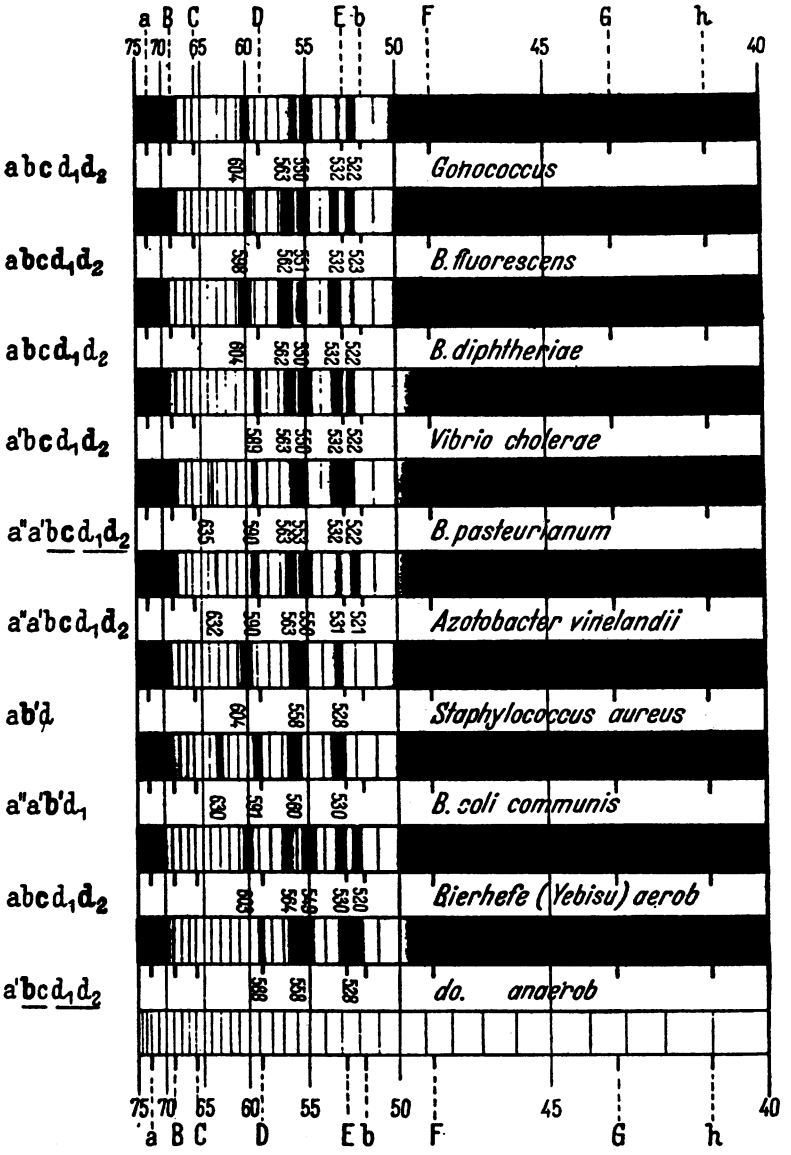

Figure 1. Representation of cytochrome spectra of bacteria (Fujita and Kodama, 14).

while the obligate anaerobes contained no cytochromes. This idea that there is a relationship between the cytochrome spectrum and the degree of aerobiosis of the bacteria has persisted (12) although more extensive studies, to be described later, have proved it to be incorrect.

Many spectroscopic examinations of the cytochromes of bacteria have been made; three examples of rather extensive studies are those of Frei *et al.* (13), Fujita and Kodama (14) and Yamagutchi (15). Figure 1 illustrates typical data. There is a great deal of disagreement among the different results reported, and an attempt to give a listing of all of the observations made on bacteria would only lead to confusion. Also, some workers did not recognize the shift

in the position of the bands of some of the bacterial cytochromes, as compared with those of mammalian tissues. Thus, for example, any band in the red region of the spectrum was sometimes reported as cytochrome a. It is not easy to see the fainter of the cytochrome bands with a visual spectroscope, and some must be judged as shadings on the side of other bands. Some of the disagreement among the different workers must result from this difficulty of seeing the bands with a visual spectroscope. For example, Yamagutchi first reported that cytochrome a or a_1 was missing from a number of bacteria; later, using thicker suspensions (15), he was able to see these bands.

The observations of the investigators mentioned above, together with those of several others (16–22), can be summed up in the following generalizations:

(a) Some bacteria showed absorption spectra in the visible region of the spectrum which are very similar to that of yeast or mammalian tissues; examples of these are some strains of *Bacillus subtilis*, and *Sarcina lutea*, *Bacillus pertussis*, *Bacillus fluorescens*, *Bacillus pyocyaneus* and some mycobacteria.[1]

(b) Cytochrome a often was found to be missing from bacteria and was replaced by cytochrome a_1 (a′), with a weak band at about 590 mμ or by cytochrome a_2 (a″), with a more intense band around 630 mμ, or by a mixture of cytochromes a_1 plus a_2. For example, *Escherichia coli* usually were observed to contain both cytochromes a_1 and a_2 although sometimes one or the other was not seen or both were claimed to be missing. Similar observations have been reported for *Azotobacter chroococcum*, whereas *Acetobacter pasteurianum* seemed to have only cytochrome a_1. Yamagutchi (15) also described a pigment with a band at 598 mμ, which he called cytochrome a′, and Chin (23) found a cytochrome a_4, with a band at 612 mμ in the reduced form, in *Acetobacter peroxydans*, which also seemed to contain cytochromes a_1 and a_2.

(c) In some bacteria, the combination of cytochromes b and c seemed to be replaced by a single strong band at about 560 mμ, usually referred to as cytochrome b_1 (or b′). Some workers suggested that the cytochrome b_1 band resulted from the fused bands of cytochromes b and c. *E. coli* and *Proteus vulgaris* are examples of bacteria which often were reported to contain cytochrome b_1 although Yamagutchi (15) thought that he saw evidence of a band of cytochrome c in these two kinds of bacteria. Fujita and Kodama (14) reported that several species of staphylococci contained cytochrome b_1.

Yamagutchi (15) commented that in all of the bacteria examined by him, either cytochrome b or b_1 was present, and in some facultative anaerobes, cytochrome b was thought to be the only visible cytochrome (24). It was suggested that this hemochromogen might be the precursor of the other cytochromes, but this suggestion was not supported by experimental evidence.

(d) No cytochromes were seen in a number of species of streptococci and pneumococci or in any of the obligate anaerobic bacteria examined. However, recently Kamen and Vernon (25) found evidence for the presence of a cytochrome with a spectrum similar to that of cytochrome c in a strain of *Chlorobium limicola*, an obligate anaerobe and photoautotroph. They also reported cytochrome oxidase and cytochrome c reductase activities in extracts of these organisms. Postgate (26) found reversibly oxidizable cytochromes in another strict anaerobe, *Desulfovibrio desulfuricans*. The generalization that cytochromes are absent from obligate anaerobic bacteria no longer holds.

B. Variations in Bacterial Cytochromes

Some of the disagreements found in the data of the various workers on cytochrome spectra might be explained as due to variations among the different strains of bacteria used or to variations with different culturing conditions. Thus, we might consider next what observations have been made concerning variations of bacterial cytochromes. It is well established (27, 28, 29) that the cytochrome spectrum of yeast grown aerobically differs from that of anaerobically grown yeast.

Chaix and Roncoli (30) and Frei *et al.* (13) described changes in the spectrum of the cytochromes of *B. subtilis* from that of a mixture of cytochromes a plus b_1 in the "resting state" to a combination of cytochromes a, b and c with growth. The rate of change of the spectrum seemed to vary with the strain of the bacteria and also was influenced somewhat by growth conditions (30). Similar variations were not

[1] The nomenclature of the bacteria is that of the authors quoted.

found with *E. coli* by Chaix and Roncoli or by Schaeffer (31) although Tamiya and Yamagutchi (16) claimed that the cytochrome a band (by which they must have meant cytochrome a_1 or a_2) was present if the *E. coli* were grown in the presence, but not in the absence, of glucose.

Moss (32) examined a strain of *E. coli* that showed an increase in the respiration rate of the cells and a more rapid appearance of cytochrome a_2 as the oxygen tension during growth was increased. He observed no correlation between the increase in the cytochrome a_2 band and the change in respiratory rate; in fact, at low oxygen concentrations, the cytochrome a_2 content increased more than did the Q_{O_2}. The cytochrome b_1 band was not seen in anaerobically grown cells, but an intense band appeared after growth for 4 hours with aeration.

Schaeffer (31) tested the effect of variations in oxygen tension during growth on the cytochrome spectrum of *Bacillus cereus*. He also seems to have observed quantitative variations in the cytochromes with change in oxygen tension, rather than variations in type of cytochromes. Cytochromes a, b and c were seen in aerobically grown cells, while cytochromes a and c were not seen in cultures grown anaerobically, and the cytochrome b band was decreased in intensity. In spite of the loss in cytochrome content, the anaerobically grown cells showed a greater rate of oxygen uptake in the presence of glucose than did those grown aerobically. Schaeffer (33) also observed that cells grown anaerobically released a porphyrin into the medium, and that, in a mutant of *B. cereus* requiring streptomycin, deprivation of streptomycin resulted in a decrease in the cytochrome content of the cells as well as a decrease in the intracellular protohemin (34).

Lenhoff and Kaplan (35) describe an unusual effect of oxygen tension on the cytochrome content of *Pseudomonas fluorescens*. They found a decrease in a cytochrome c-like pigment when the bacteria were grown under high oxygen tension (aerated cells), as compared with cells grown without aeration.

Also variations in the composition of the medium have been shown to produce changes in the cytochromes and in the oxidative metabolism of bacteria. Specifically, the changes resulting from the growth of *Aerobacter indologenes* in an iron deficient medium were investigated by Waring and Werkman (36). The spectrum of cytochromes a_1 and b_1 was seen in cells grown on an adequate medium, while iron deficient cells showed no cytochrome bands. However, the deficient cells grown aerobically had the same respiratory rate in the presence of glucose as did the normal cells although the rate of oxygen uptake of the deficient cells was decreased when lactate, pyruvate or acetate was oxidized. Pappenheimer (37) found with *Corynebacterium diphtheriae* that within a certain range of iron concentration in the medium, the addition of iron to the medium produced an increase in the cytochrome content of the cells. Gary and Bard (38) observed what appear to be changes in the cytochrome system of *B. subtilis* grown on media of different compositions.

Sometimes variations in bacterial cytochromes have been observed under constant culturing conditions. Tissières (39) has described differences in the cytochrome a_2 content of different variants of a strain of *Aerobacter aerogenes* grown under similar conditions. He was also able to decrease the cytochrome a_2 in these bacteria to very low levels by growth in an iron deficient medium, but this decrease did not always affect the respiration rate.

Keilin (40) found that the cytochrome a_2 content of *Acetobacter pasteurianum* varied with the culture medium and the age of the culture as well as with the strain of the organism. Sometimes no cytochrome a_2 was evident; cytochrome a_2 appears to be the most variable of the cytochromes.

It is clear from the observations of the variations of the bacterial cytochromes that great caution must be exercised in basing conclusions upon changes in the cytochrome content of the bacteria. We have even observed (41) that when washed cells of *Azotobacter chroococcum* stand in buffer overnight, the cytochrome content will increase about twofold, as judged by the increase in height of peaks in the absorption spectrum.

So far, these observations on bacterial cytochromes, although interesting, have not given us any information concerning the relationship of the cytochromes to the bacterial respiratory systems. It is difficult to explain why large decreases in the content of the cytochromes often do not result in a lowered respiration rate. Further studies of these changes, using more sensitive techniques, might lead to a better understanding of the functioning of these pigments.

C. *Difference Spectra*

Spectroscopic observations of cytochrome bands are subject to the limitations already discussed in Section A. Frei *et al.* (13), in some instances, photographed the absorption spectra of bacteria to fix more accurately the positions of the bands; and Euler, Fink and Hellström (42) observed similarly the difference between the oxidized and reduced forms of the pigments in yeast, using strong H_2O_2 to keep the pigments oxidized long enough to photograph them. Chaix and Fromageot (43) attempted to make spectroscopic measurements more quantitative by photographing the spectra of the pigments in the oxidized and reduced forms after passing through oxygen or nitrogen, then analyzing the photographs obtained by means of a microphotometer. The only kind of bacteria they examined was a strain of *Propionbacterium pentosaceum*, which they found to contain cytochrome b, but no cytochrome c.

It is not possible to measure the spectra of the cytochromes in turbid suspensions of whole cells or homogenates by spectrophotometric techniques because of the great light-scattering properties of the suspensions. However, we have been able to measure the difference spectra of the cytochromes of intact, respiring bacteria (41), using the sensitive spectrophotometric techniques developed by Chance (44, 45).[2] The respiring bacteria were aerated; then the changes in optical density at a series of wavelengths from 380–650 mμ were recorded with great sensitivity[3] as the bacteria used up the oxygen in the solution and the pigments changed from the steady-state oxidized to the reduced form. Conditions have been found for each organism[4] which insure that their light-scattering properties remain constant while the pigments change from the oxidized to the reduced form; then the turbidity does not interfere with the measurements of the difference in optical density of the two forms. The respiration was followed simultaneously by means of a platinum microelectrode; the reduction of the respiratory enzymes coincides with the termination of oxidase activity as the oxygen concentration reaches zero. The optical density increments, plotted against the wavelengths, give the difference spectra of the pigments present; figures 2 to 6 are examples of the difference spectra of several kinds of bacteria. Figure 7 shows the difference spectrum of yeast for comparison.

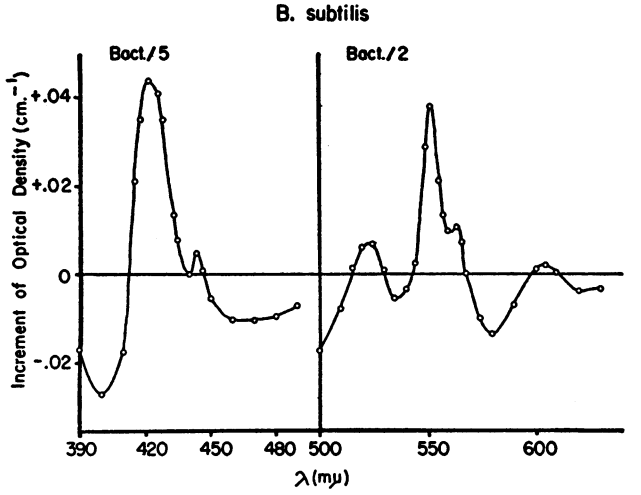

Figure 2. Difference spectrum of *Bacillus subtilis* (Smith, 41).

From the difference spectra, the positions of the peaks in the visible region of the spectrum are definitely established, and the method allows

[2] As shown by Chance, the light-scattering by the turbid suspensions does not interfere with measurements of the changes in optical density as the pigments go from the oxidized to the reduced forms if the light-scattering is the same in the two forms.

[3] The optical density changes can be measured with an error of 2×10^{-4} in optical density.

[4] Conditions are described in reference (41).

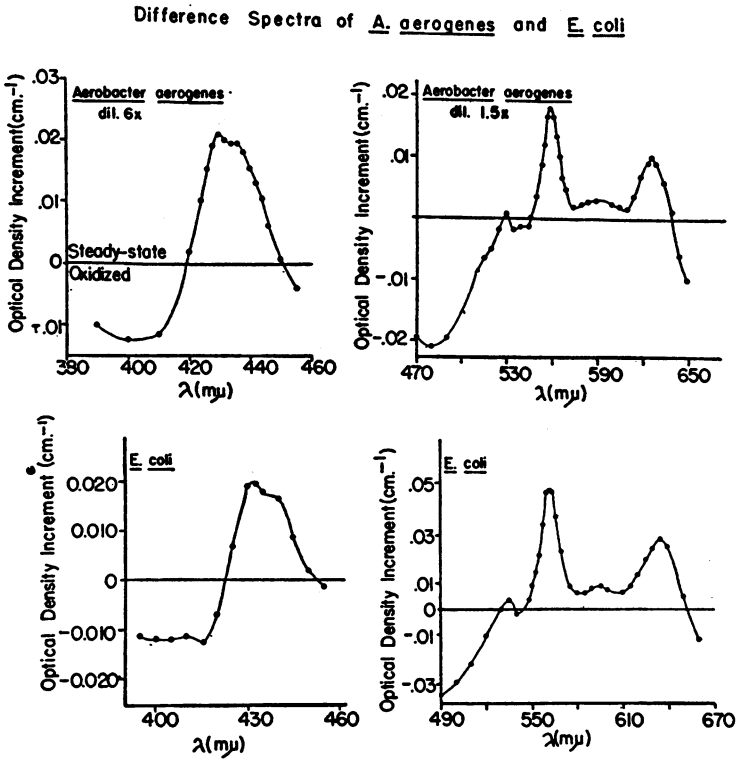

Figure 3. Difference spectra of *Aerobacter aerogenes* and *Escherichia coli*. The suspension of *E. coli* was diluted twice for measurements in the visible region of the spectrum and six times for measurement in the Soret region (Smith, 41).

extension of the observations into the violet region (Soret region), which has not been explored previously in observations of bacterial cytochromes.

Table 1 summarizes the data on the positions of the cytochrome peaks from measurements of the difference spectra. The data show that:

(a) *B. subtilis* and *Sarcina lutea* have cytochrome spectra qualitatively similar to that of yeast and mammalian tissues. The spectrum of *Staphylococcus albus* [*Micrococcus pyogenes* var. *albus* (Rosenbach) Schroeter] is similar in the visible region but shows no peak in the Soret region corresponding to cytochrome a_3. Three strains of *S. albus* examined by us showed no evidence of cytochrome b_1, in disagreement with the observations of Fujita and Kodama (14).

(b) The absence of cytochrome a in a number of bacteria is established, and the small peak of cytochrome a_1 in *Acetobacter pasteurianum* and *Azotobacter chroococcum* could always be distinguished. Warburg *et al.* (46) could see the cytochrome a_1 band only in strong (25–30%) suspensions of *Acetobacter pasteurianum*, and Negelein and Gerischer (47) did not see it at all in *Azotobacter chroococcum*. The combination of cytochromes a_1 plus a_2 is present in several bacteria tested, and cytochrome a_2 was never seen in the absence of cytochrome a_1. After a number of transfers, the azotobacter changed so that the cytochrome a_2 peak was no longer visible; this was accompanied by no change in the Soret region of the spectrum. Apparently the cytochrome a_2 has very little absorption in the violet region of the spectrum. Data on the reaction of this cytochrome with cyanide[5] and with carbon monoxide (48) corroborate this finding, which agrees with the suggestion of Lemberg and Wyndam (49) that cytochrome a_2 resembles a biliviolin hemochromogen and thus should have no peak in the Soret region (50). Our observations also agree with the finding of Tissières (39) that the loss of the cytochrome a_2 peak had

[5] Smith, L., unpublished data.

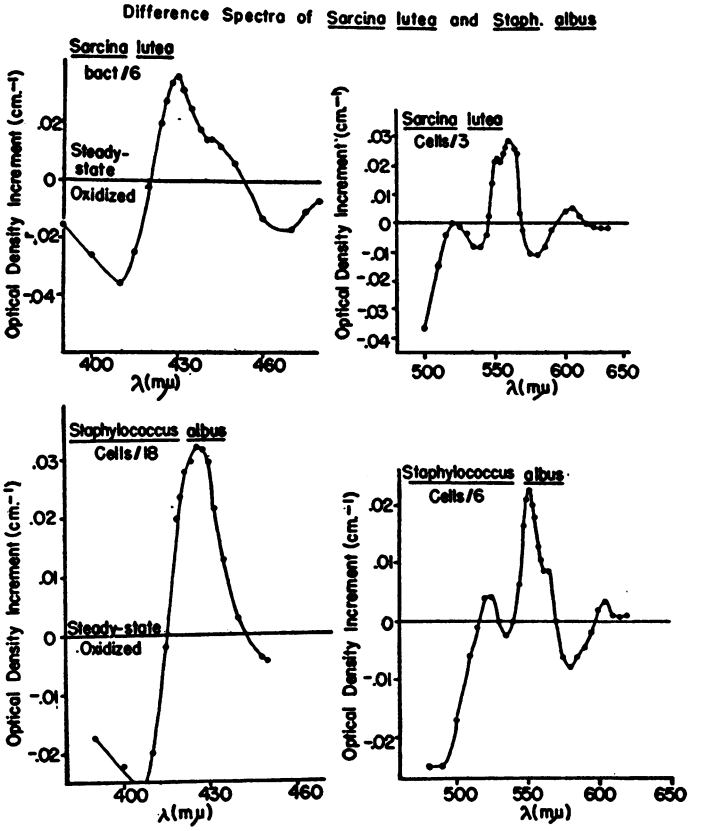

Figure 4. Difference spectra of *Sarcina lutea* and *Staphylococcus albus* (Smith, 41).

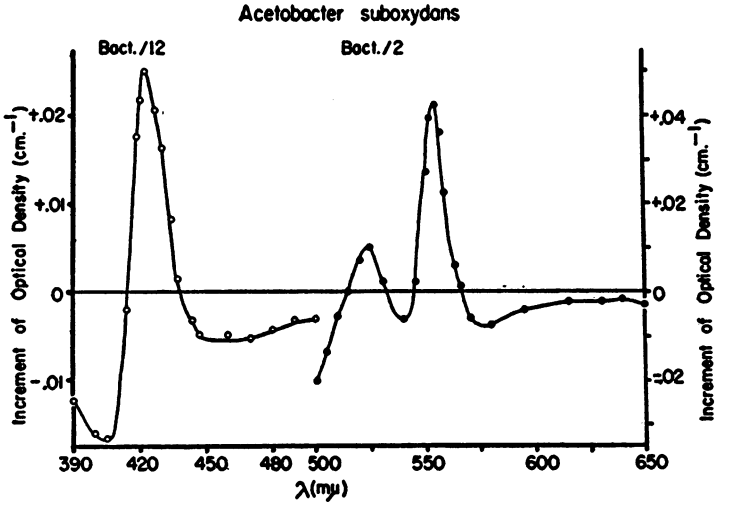

Figure 5. Difference spectrum of *Acetobacter suboxydans* (Smith, 41).

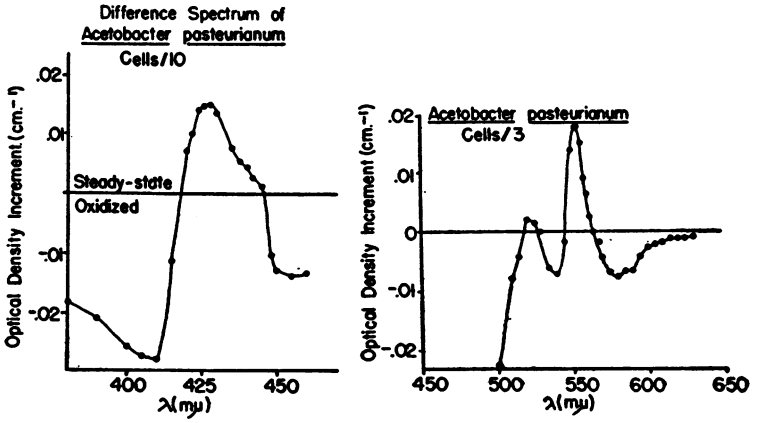

Figure 6. Difference spectrum of *Acetobacter pasteurianum* (Smith, 41).

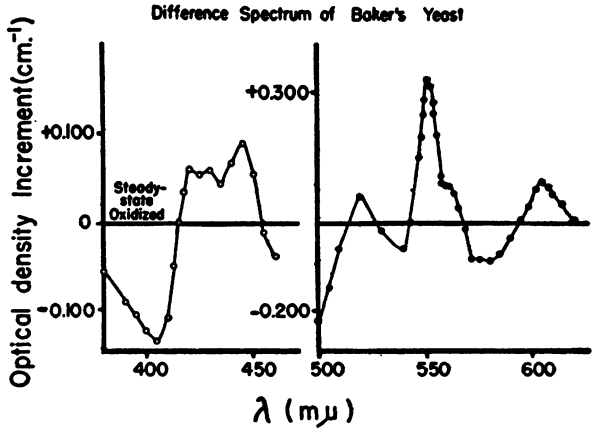

Figure 7. Difference spectrum of baker's yeast. Cells undiluted for measurements in the visible region of the spectrum and diluted 4.5 times for measurements in the Soret region (Chance, 45).

no effect on the respiration rate of these bacteria in the presence of glucose (see table 2).

The loss of the cytochrome a_2 peak of the azotobacter was the only qualitative change in the bacterial cytochromes seen during our experiments, where a great effort was made to maintain constant culturing conditions.

(c) In one species of bacterium, *Acetobacter suboxydans*, no evidence of cytochrome a nor of cytochromes a_1 or a_2 was obtained. This observation makes these bacteria unique among the bacteria that contain cytochromes.

(d) In several bacteria, cytochrome b_1, with a peak at 560 mμ, is present. That this peak was not due to the fused peaks of cytochromes b plus c was demonstrated in *P. vulgaris* by warming the bacteria at 70 C for 10 minutes, a procedure which will destroy cytochrome b. The cytochrome b_1 peak remained unchanged after warming.

(e) In most of the bacteria examined the Soret peak is dominated by a peak around 430 mμ, which must be that corresponding to cytochrome b or b_1. However, the supposition, mentioned above, that cytochrome b or a similar hemochromogen is always present in bacteria is not substantiated by our work.

(f) The band at 554 mμ seen in the two strains of acetobacter seems to belong to a pigment different from any cytochrome previously described. It is the only cytochrome component apparent in *Acetobacter suboxydans*.

(g) Almost any possible assortment of the different cytochromes can be found in the group

TABLE 1

Peaks in difference spectra of bacterial cytochromes
(Wavelengths in millimicrons)

BACTERIA	VISIBLE						SORET	
	α				β		γ	
Bacillus subtilis		604		564	552	523	444	422
Staphylococcus albus		604		565	552	523		427
Sarcina lutea		605		562	552	523	444	430
Micrococcus lysodeikticus		600			552	520	440	432
Aerobacter aerogenes	628		592	560		530	435	430
Escherichia coli	630		593	560		533	437	432
Proteus vulgaris	630		595	560		533	440	430
Azotobacter chroococcum	628		590	560	552	530	440?	428
A. chroococcum after several transfers			590		552	522		427
Acetobacter pasteurianum			588		554	523	445	428
Acetobacter suboxydans					554	525		422
Pseudomonas fluorescens			580	560	552	523		424
Streptococcus faecalis	No cytochromes							
Yeast		605		563		525	445	426
Heart muscle particles		605		563		525	445	426

Peaks at 628–630, 604–605 mμ and at about 590 mμ correspond to the α peaks of cytochrome a_2, a and a_1, respectively. The 564–565 mμ peak and that at 560 mμ have previously been designated as the α peak of cytochromes b and b_1. The identity of the peaks at 552 and 554 mμ is not established. The peaks around 520–530 mμ represent the β bands of the cytochromes present, and those between 442–444 mμ, the γ or the Soret bands. Data of Smith (41).

of bacteria examined, with the exception that cytochrome a does not occur together with cytochromes a_1 and a_2, and cytochrome a_2 does not occur in the absence of cytochrome a_1.

(h) No obvious relationship was observed between the cytochrome components and the degree of aerobiosis of the cell.

(i) *Streptococcus faecalis*, although it can respire, contains no cytochromes. The difference spectrum obtained is typical of that of a flavoprotein compound.[5]

Quantitative Aspects

From the difference spectra, several quantitative calculations can be made, expressing the cytochromes in terms of OD at 500 mμ (turbidity) and of rate of oxygen uptake. These ratios for the bacteria can be compared with similar "Figures of Merit" described by Chance (51) for heart muscle particles and yeast, as seen in table 2. K_1 gives a measure of the respiration rate at 25 C related to turbidity (dry weight) and with a standard curve relating optical density to dry weight can be converted to the conventional Q_{O_2}. The values of K_1 for bacteria vary widely from 0.37 for *Staphylococcus albus* to 8.6 for *Acetobacter suboxydans*. K_3 is the ratio of the main Soret peak to the turbidity and gives an indication of the ease of studying the bacterial pigments with spectrophotometric methods. Most bacteria are superior to yeast in this respect, and a strain of *Azotobacter vinelandii* has a K_3 of 30,[5] which compares favorably with the value for heart muscle particles. Calculations based on the K_3 values show that the cytochrome content of *Aerobacter aerogenes* and *Azotobacter chroococcum*, expressed as the increase in optical density on reduction at the main Soret peak per mg dry weight, is ten times greater than that of yeast.

The values of K_4 relate the respiratory rate to the cytochrome content and thus give an indication of the activity of the bacterial cytochrome systems. From the K_4 values, actual turnover numbers have been calculated for the cytochrome a_3 of *B. subtilis* and the cytochrome a_1 of *Acetobacter pasteurianum*, using the values of $\Delta\epsilon$ measured by Chance (52). The data show that many of the bacterial cytochrome systems work with great efficiency, having high turnover

TABLE 2

"Figures of Merit" for bacteria, yeast and heart muscle particles

	SUBSTRATE	K_1	K_3	K_4		TURNOVER NUMBER
				a	b	
Yeast	alcohol	0.6	3	186	186	65
Heart muscle particles	succinate	4.0	80–100	80	80	29
Acetobacter pasteurianum	alcohol	3.9	3	1,291	4,500	620
Acetobacter pasteurianum	glucose	0.2	5	39		
Aerobacter aerogenes	lactate	2.8	18	160	218	
Azotobacter chroococcum	glucose	2.4	13	185		
Azotobacter chroococcum after changing	glucose	2.6	11	245		
Staphylococcus albus	glucose	0.4	4	89		
Sarcina lutea	succinate	1.6	2	847	2,130	
Escherichia coli	succinate	3.6	9	411	454	
Micrococcus lysodeikticus	lactate	1.8	2	853		
Acetobacter suboxydans	alcohol	8.6	6	1,530		
Bacillus subtilis	glucose	0.7	8	82	760	76
Pseudomonas fluorescens	glucose	1.7	8	209		

$$K_1 = \frac{O_2 \text{ uptake } (\mu M/\text{sec})}{\text{OD at } 500 \text{ m}\mu} \qquad K_4 \text{ (a)} = \frac{O_2 \text{ uptake } (\mu M/\text{sec})}{\Delta \text{ OD main Soret peak}}$$

$$K_3 = \frac{\Delta \text{ OD Soret peak}}{\text{OD at } 500 \text{ m}\mu} \qquad K_4 \text{ (b)} = \frac{O_2 \text{ uptake } (\mu M/\text{sec})}{\Delta \text{ OD } 440 \text{ or } 444 \text{ m}\mu}$$

The K_1, K_3 and K_4 values correspond to those described by Chance (51) for heart muscle particles. They were calculated from measurements of oxygen uptake at 25 C (expressed as μM/sec), optical densities at 500 mμ, and from the data in the difference spectra of each of the different kinds of bacteria.

The turnover numbers were calculated from the following formula:

$$TN = K_4 \times 4 \times \Delta\epsilon$$

using the values for $\Delta\epsilon$ obtained by Chance (52).

Data are from Smith (41).

numbers. The cytochrome systems of the two strains of acetobacter are particularly active.

The spectrophotometric studies of bacteria have given us information about the distribution of the different kinds of cytochromes and about some quantitative aspects of their activities in the bacterial respiratory systems. We know that the cytochrome components of most bacteria are different from those of mammalian tissues, and that the bacterial systems can show high rates of turnover. This kind of investigation, however, does not tell us anything about the functions of the individual pigments.

III. FUNCTIONS OF THE BACTERIAL CYTOCHROME SYSTEMS

A. Oxidase Activity

The work of Keilin and Hartree (53) established some of the properties of the oxidase of heart muscle, which they showed to be cytochrome c oxidase, the enzyme which catalyzes the oxidation of cytochrome c by oxygen. The cytochrome oxidase–cytochrome c system can oxidize a number of substances, among them the "Nadi" reagent (dimethyl *p*-phenylenediamine plus α-naphthol), *p*-phenylenediamine and hydroquinone, and the oxidase activity is inhibited in the same manner as is cellular respiration by cyanide, azide, sulfide and carbon monoxide, the inhibition by the latter being reversed by light. The enzyme is attached to insoluble cellular particles and has resisted purification; thus, it has been studied as part of the complex of enzymes attached to the particles.

These same reactions described for the heart muscle cytochrome oxidase–cytochrome c system have been investigated in bacteria and bacterial extracts. The ability of some, but not all, bacteria to oxidize the "Nadi" reagent has been observed,

and bacterial extracts show variable abilities to oxidize *p*-phenylenediamine and hydroquinone (13, 54, 55, 56). However, the increased oxygen uptake observed on addition of *p*-phenylenediamine or hydroquinone to several kinds of bacteria was not inhibited by carbon monoxide (55). Unfortunately, the "Nadi" reaction is not specific for the cytochrome c oxidase system (57, 58), and *p*-phenylenediamine and hydroquinone can be oxidized also by peroxidases. Thus, the oxidation of these substances cannot be taken as conclusive evidence for the presence of cytochrome c oxidase.

Many observations have been made also on the effect of cyanide, sulfide, azide and carbon monoxide on the respiratory activities of bacteria (14, 59, 60). In some bacteria all of these substances have been observed to inhibit respiration, and Fujita and Kodama (14) found that with some bacteria the partition coefficient with carbon monoxide and oxygen is similar to that of yeast. On the other hand, the inhibition of respiration by carbon monoxide in a number of organisms is only slightly or not at all reversed by light. In other bacteria, not all of these inhibitors are effective. Again it should be pointed out that these reagents are not specific for cytochrome c oxidase since some will react with other heavy metal enzymes. Also care must be taken in interpreting results obtained with these inhibitors. For example, cyanide is bound readily by acids such as pyruvic or oxalacetic, and the inhibition of enzymes like cytochrome oxidase by azide is dependent upon pH.

In a number of instances, observations on the effects of inhibitors on the respiration of bacteria seem to indicate that the cytochrome c oxidase system could not be present. In those bacteria, such as *E. coli*, where the carbon monoxide inhibition of respiration is not light sensitive, or in bacteria in which the respiration is not inhibited by cyanide or sulfide, the presence of a different enzyme than cytochrome c oxidase is indicated. For example, Chaix and Fromageot (61) found that the rather small respiration of *P. pentosaceum* in the presence of glucose was not inhibited by 10^{-6} to 10^{-3} M H_2S or by 95% CO-5% O_2. Also, they claim that the oxidation and reduction of the endogenous cytochromes were not inhibited by any concentration of cyanide or H_2S although there was a 45% inhibition of the respiration by cyanide. Chaix and Kuin (62) observed in addition that H_2S did not inhibit the oxidation of the cytochromes of *B. subtilis* by air. Thus, they concluded that a different kind of oxidase was present in these bacteria. On the other hand, Gerard (63) found that the respiration of a strain of *S. lutea* was not inhibited by cyanide, but that in the presence of cyanide the cytochromes remained in the reduced state. We could not repeat his observations with the strain of *S. lutea* investigated in our laboratory. Spectroscopic ob-

TABLE 3

Cytochrome c oxidase activity of heart muscle particles, yeast and bacterial extracts, expressed on the basis of the increase in optical density at 605 mμ on reduction (67)

BACTERIA	CYTOCHROME OXIDASE ACTIVITY 1ST ORDER VELOCITY k (SEC^{-1}) × DIL.	Δ OPTICAL DENSITY 605–580* mμ × DIL.	CYTOCHROME OXIDASE/ OPTICAL DENSITY
Heart muscle particles	60	0.23	262
Yeast extract	10	0.033	303
Bacillus subtilis	0.048	0.023	2
Staphylococcus albus	0.046	0.005	9
Sarcina lutea	0.002	0.005	0.4
Azotobacter chroococcum	essentially 0		
Escherichia coli	essentially 0		
Aerobacter aerogenes	essentially 0		
Acetobacter pasteurianum	essentially 0		
Micrococcus lysodeikticus	essentially 0		

The results recorded above for bacteria were obtained with extracts prepared in the sonic vibrator.

* This value represents the difference between the peak in the difference spectrum at 605 mμ and the trough at 580 mμ, and is thus a measure of the content of cytochrome a in the different preparations. The cytochrome oxidase activity is related to the content of cytochrome a in each case.

servations of the reactions of the cytochromes with cyanide and carbon monoxide will be discussed in the following section.

The direct test for cytochrome c oxidase activity is the observation of the rapid oxidation of reduced added cytochrome c, provided that cytochrome c peroxidase activity is absent. Yamagutchi (55) claimed that he observed the oxidation of reduced cytochrome c by an extract of *B. pyocyaneus*, but he does not state the time required for the oxidation. Recently, Vernon and Kamen (64) have reported the oxidation of reduced cytochrome c by broken cell suspensions of *Rhodospirillum rubrum*, but the reaction seems to be a very slow one. They describe in addition a photochemical oxidation of cytochrome c by extracts of these organisms.

Keilin and Harpley (65) tested for cytochrome c oxidase activity in crushed cells of *E. coli commune* and found that reduced cytochrome c was not oxidized by the preparation. The reactions with oxygen seemed to take place only through the cytochrome system of the organisms. As early as 1933 (66), Keilin had suggested that *E. coli* has a different type of oxidase from that of yeast or heart muscle.

We have extended the work of Keilin and Harpley by testing the ability of broken cell suspensions of eight different kinds of bacteria to oxidize reduced cytochrome c (67). Table 3 shows that no significant cytochrome c oxidase activity was observed in any of the bacteria tested, even in *B. subtilis*, which has a pigment with the spectral characteristics of a cytochrome of type a_3 (Section III, C). The oxidases of these bacteria are different from that of heart muscle and yeast. Some developments which have finally led to a better understanding of the cellular oxidases will be discussed in Section III, C.

B. Reactions with Oxygen and with the Reducing Systems

Investigations of the time sequence of reaction of the cytochromes could give some information about the order of reaction of these pigments. Actually, it is very difficult to judge time differences in the appearances and disappearances of the cytochrome bands using a visual spectroscope since the bands are not of equal intensities.

Yamagutchi (15) reported that after shaking with air, then allowing some bacteria to become anaerobic, the cytochrome c plus a (or a_1) spectral bands appeared first and reached their maximum intensities rapidly, then the band of reduced cytochrome b appeared and developed its maximum intensity slowly. He also claimed that if cyanide (10^{-4} M) was added to the bacterial suspensions, the cytochrome c band was seen to appear immediately, then the reduced cytochrome b band appeared later. Yamagutchi's results unfortunately are confusing since he did not state which of the different bacteria he was observing, and not all of the bacteria he reported to contain cytochrome c have this pigment. He warmed some *S. lutea* for 60 minutes at 52 C to decrease the dehydrogenase activity, then added varying concentrations of cyanide; this treatment emphasized the time differences in reduction and oxidation of cytochromes a and c as compared with cytochrome b. These observations make it appear as if cytochromes a and c might participate in a different sequence of reactions from cytochrome b.

Tissières (39) observed respiring cells of *Aerobacter aerogenes* with a spectroscope at low temperatures, so that the reaction rates were slowed down, in an attempt to determine the sequence of oxidation and reduction of the different cytochromes. These bacteria showed a faint band of cytochrome a_1 and strong bands of cytochromes a_2 and b_1. He concluded that as the oxygen tension reached zero, cytochrome a_2 seemed to be reduced after cytochrome b_1, which he believed meant that cytochrome a_2 is the cytochrome nearer to oxygen in the chain of reactions. However, Chance (48) has shown that the pigment which reacts with oxygen should be the first to be reduced as the oxygen concentration goes to zero since it is the only component directly responsive to oxygen tension. Chance, using a rapidly recording spectrophotometer, found that cytochrome b_1 is reduced more slowly than cytochrome a_2 as the oxygen tension reaches zero, in contrast to the result of Tissières, but in agreement with his conclusion that cytochrome a_2 is nearer to the oxidase component than is cytochrome b_1. Still, the great variability of cytochrome a_2 in cells must be remembered, as well as the lack of correlation between the cytochrome a_2 content and the respiratory activity of the bacteria. Also, Tissières (68) prepared cell-free extracts of *Aerobacter aerogenes* which could oxidize succinate, glucose and pyruvate, and an active succinic oxidase system was prepared by ammonium sulfate fractiona-

tion. All the active fractions contained cytochromes a_1 and b_1, but cytochrome a_2 apparently was lost during the preparation. This sort of observation argues against postulating that cytochrome a_2 acts as the respiratory enzyme of these bacteria.

C. Reactions of Bacterial Cytochromes a, a_1, a_2 and a_3

Keilin and Hartree (3) had observed that cytochromes a, b and c of heart muscle do not react with cyanide or carbon monoxide and they were not rapidly autooxidizable. Thus, none of these cytochromes would be the oxidase of these cells. When they examined these reactions of the cytochromes of heart muscle suspensions in the Soret region of the spectrum, they were able to distinguish cytochrome a_3, the bands of which are superimposed upon those of cytochrome a. As discussed previously, cytochrome a_3 is responsible for the main part of the Soret peak of the cytochrome a plus a_3 combination; and, in contrast to cytochrome a, cytochrome a_3 reacts with cyanide and carbon monoxide and is autooxidizable. Since the main part of the visible band of cytochromes a plus a_3 is due to cytochrome a, the reactions of cytochrome a_3 with cyanide and carbon monoxide do not show up well in the visible region of the spectrum.

Before the reactions of cytochrome a_3 were established, a number of investigations had been made on the reactions of bacterial cytochromes with carbon monoxide and cyanide; some of these reactions can be observed in the visible region of the spectrum. Studies of the reactions with carbon monoxide have been important in the development of our knowledge of these enzymes.

Any enzyme with which carbon monoxide reacts in competition with oxygen would be a cellular oxidase. The competitive inhibition of some respiratory enzymes by CO is reversed by light, and it was this property that was used (4) to measure the photochemical action spectrum of the respiratory enzymes of *Torula utilis* and *Acetobacter pasteurianum*. It was concluded that the carbon monoxide spectrum of the respiratory enzyme in the two organisms is the same, with absorption peaks at 590, 540 and 430 mμ. Until recently, photochemical action spectra have been obtained for only a few other types of cells: baker's yeast, heart muscle suspension and chick embryo, all of which seemed to have the visible peak at 590 mμ, but there is some disagreement about the Soret peaks. In our laboratory, Castor and Chance[6] have devised a simpler method for determining the action spectra, using more wavelengths of light. They found that the action spectra of the enzymes of baker's yeast, heart muscle and *B. subtilis* all have Soret peaks at 431 mμ.

When Warburg *et al.* (46) were able to see the α-band of cytochrome a_1 in strong suspensions of *Acetobacter pasteurianum* and observed that the band seemed to shift to about 593 mμ in the presence of carbon monoxide, they concluded that they had made a direct observation of the absorption bands of the respiratory enzyme. They also saw that under aerobic conditions in the presence of cyanide, this band seemed to disappear and to be replaced by a band in the red at 639 mμ; thus, the pigment appeared to be autooxidizable, an observation in agreement with their conclusion that they were observing the respiratory enzyme. They were unable to see a similar band in yeast, but believed that it might be obscured by the band of cytochrome a. They finally suggested that the bands at 589 and 639 mμ might belong to two different hemin enzymes, rather than to the oxidized and reduced forms of the respiratory enzyme since, under some conditions, both bands were seen together.

In *Azotobacter chroococcum*, Negelein and Gerischer (47) saw the absorption band of cytochrome a_2 but not that of a_1. They concluded that in these bacteria carbon monoxide and cyanide and oxygen all react with cytochrome a_2, which they apparently designated as the respiratory enzyme of these cells.

Other observations made on the reactions of bacterial cytochromes a_1 and a_2 are not in agreement with those of the Warburg group. As mentioned above, Keilin (40) found *Acetobacter pasteurianum* to be a "polymorphic" organism, and the effect of carbon monoxide on the band at 595 mμ was variable. Also he observed no change in the cytochrome a_1 band of *Azotobacter chroococcum* in the presence of carbon monoxide, while Fujita and Kodama (14) thought that the cytochrome a_1 of this organism reacted with carbon monoxide, but that it was not autooxidizable. The bands of cytochrome

[6] Castor, L. N., and Chance, B., unpublished observations.

a_1 seen in *E. coli* and *P. vulgaris* also do not change in the presence of carbon monoxide (14, 17). Most of the observations of the above workers agree that cytochrome a_2 in the different bacteria reacts to give a spectral change in the presence of carbon monoxide or cyanide, but Yamagutchi (15) could see no reaction of the cytochrome a_2 in a number of bacteria, including *Proteus mirabilis*, with cyanide. Also Tissières (39) observed that in the beginning of his work with *Aerobacter aerogenes*, the cytochrome a_2 reacted with cyanide, but after working with the culture for several months the bacteria changed so that cyanide no longer affected the cytochrome a_2 band but still strongly inhibited respiration. Chin (23) reported that the cytochromes a_1, a_2 and a_4 of *Acetobacter peroxydans* could all react with cyanide and carbon monoxide as shown by spectral changes on addition of these substances.

Obviously a lot of work has been done on the reactions of cytochromes a_1 and a_2 with respiratory inhibitors like cyanide and carbon monoxide, on the assumption that such observations might reveal pertinent information about the nature of the cellular oxidases. The variable effects observed for the reactions of cytochrome a_1 with these inhibitors and the variability of cytochrome a_2, together with the lack of correlation between the cytochrome a_2 content and the respiration of the bacteria, have left the subject of the function of these cytochromes as respiratory enzymes in confusion.

The observations of Keilin and Hartree (3) on the reactions of heart muscle cytochrome a_3 with substances such as carbon monoxide and cyanide indicated that this was the respiratory enzyme of these cells, and they demonstrated similar reactions in yeast and *B. subtilis* (69). However, they were not able to show the dissociation of the cytochrome a_3-carbon monoxide compound by light. Keilin suggested that cytochromes a and a_3 were invariably associated (65) and that in those bacteria which contained cytochrome a, cytochrome a_3 was the oxidase. In bacteria devoid of cytochrome a (and thus presumably of cytochrome a_3) which showed strong inhibition of respiration by cyanide and light sensitive inhibition of respiration by CO, he suggested that one of the other cytochromes might have the function of cytochrome a_3, or that the true oxidase might be an entirely different enzyme. Only recently, the work of Chance has served to resolve some of the problems in the relationships between Warburg's respiratory enzyme, Keilin's cytochrome a_3 and cytochrome c oxidase, as well as cytochromes a_1 and a_2.

Chance (48) devised a method for directly recording, by means of a sensitive spectrophotometer, the carbon monoxide difference spectra (difference between the spectrum of the carbon monoxide compound and the reduced pigment) of the cytochromes in whole cells or cellular extracts. By an ingenious method (5), he also measured the photodissociation spectra of the carbon monoxide compounds of the oxidases of heart muscle particles, baker's yeast, *B. subtilis*, *Acetobacter pasteurianum* and *S. albus* with great accuracy. These are illustrated in figures 8 and 9. The spectra obtained by the two methods were in agreement and led to the following interesting conclusions:

(a) The Soret peak of the carbon monoxide compound of cytochrome a_3 in baker's yeast and heart muscle is at 430 mμ; this agrees with the observations of Keilin and with the action spectrum for *Torula utilis*, as determined by Warburg (4) and for baker's yeast, measured by Castor and Chance.[6] Thus, the cytochrome a_3 of yeast and heart muscle is identified unequivocally on a spectroscopic basis with cytochrome c oxidase and the respiratory enzyme. Both yeast and heart muscle show strong cytochrome c oxidase activity.

(b) In *B. subtilis*, a carbon monoxide com-

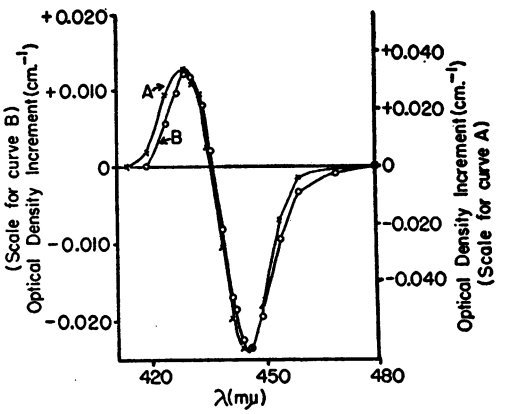

Figure 8. Photodissociation difference spectra of heart muscle particles (curve A) and baker's yeast (curve B).

The difference spectra were obtained by illuminating the carbon monoxide compounds with red light (Chance, 5).

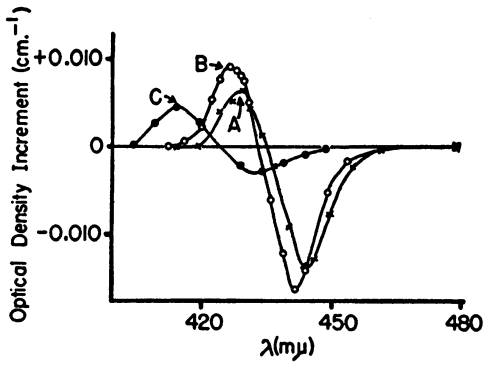

Figure 9. Photodissociation spectra of *Bacillus subtilis* (curve A), *Acetobacter pasteurianum* (curve B), and *Staphylococcus Albus* (curve C) (Chance, 5).

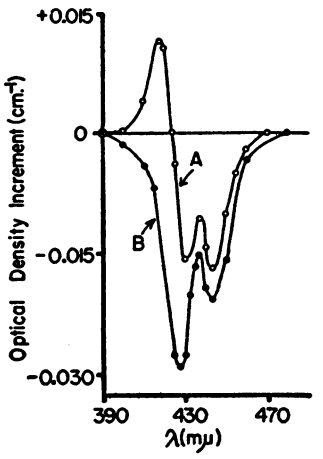

Figure 10. Carbon monoxide difference spectra of *Proteus vulgaris* (curve A) and *Aerobacter aerogenes* (curve B).

The curves represent the difference between the spectra of the carbon monoxide compounds of the cytochromes present and the reduced cytochromes (Chance, 48).

pound of type a_3 was also observed; that is, the peaks in the spectrum of the carbon monoxide compound are at the same wavelengths as that of yeast and heart muscle. However, the ratio of the Soret peak to the visible peak of the *B. subtilis* enzyme is different from that of the other two. As has been mentioned, the *B. subtilis* enzyme cannot catalyze the oxidation of reduced cytochrome c, so it is different in activity also. Also Chaix and Kuin found that the oxidase of *B. subtilis* is not inhibited by H_2S (62).

(c) In *Acetobacter pasteurianum*, the peak of the Soret spectrum of the carbon monoxide compound is displaced from that of the cytochrome a_3-carbon monoxide compound by several mμ (see figure 9); thus, the respiratory enzyme of *Acetobacter pasteurianum* differs from that in heart muscle and yeast although the method of Warburg and co-workers did not detect this difference. Chance has defined this respiratory enzyme as cytochrome a_1; the peaks of its carbon monoxide compound are at 590, 540 and 427 mμ.

(d) A new type of respiratory enzyme was observed in *S. albus*; the Soret peak of its carbon monoxide compound is at 416 mμ. The nature of this compound will be discussed in the following section.

(e) The Soret spectra of the carbon monoxide compounds of the other bacteria investigated seemed to be those of mixtures of compounds. *P. vulgaris*, for example, seems to contain both cytochrome a_1 and the new type of enzyme observed in *S. albus*. *Aerobacter aerogenes*, *E. coli* and *Azotobacter chroococcum* contain a more complicated mixture in which the components are not readily resolved but are believed to be a combination of cytochromes a_1 and a and the new respiratory enzyme (see figure 10). In these bacteria containing cytochrome a_2, a larger change in the visible region of the spectrum on addition of carbon monoxide is observed than in the Soret region. This is very different from the relationship in bacteria which contain only cytochrome a_1 or a_3 or the pigment of *S. albus*.

From the direct measurement of the kinetics of photodissociation of the carbon monoxide compounds of the oxidases of yeast, heart muscle, *B. subtilis*, *Acetobacter pasteurianum* and *Achromobacter fischeri*, Chance (52) was able to calculate the molecular extinction coefficients of these pigments (see tables 4 and 5). The values will be useful in further investigations of these enzymes.

The work of Chance has made it clear that the respiratory enzymes or cytochrome oxidases comprise a family of enzymes that may have a common enzymatic function but still differ considerably in their chemical natures and modes of action. This is not surprising since such variations occur in other hemoproteins, such as the hemoglobins or the catalases. The substrate of the oxidase in yeast and heart muscle is cytochrome c, and the enzyme should be referred to as cytochrome c oxidase. The substrates of the other oxidases are as yet unknown.

TABLE 4

Molecular extinction coefficients of γ- and α-bands of the carbon monoxide compounds of yeast, heart muscle and several bacteria

	CYTOCHROME a_3			CYTOCHROME a_1	NEW RESPIRATORY ENZYME
	Heart muscle	Yeast	*Bacillus subtilis*	*Acetobacter pasteurianum*	*Achromobacter fischeri*
α	11.4 (589 mμ)	12 (589 mμ)	12 (589 mμ)	12 (589 mμ)	5 (546 mμ)
γ	110* (430 mμ)	115 (430 mμ)		81 (430 mμ)	

* Assuming that the relationship of the γ- to the α-band in the action spectrum is the same as that in yeast.

TABLE 5

Δε values for the difference between the reduced and oxidized forms of cytochromes a_1 and a_3

CYTOCHROME a_3	CYTOCHROME a_1	Δε OXIDIZED MINUS REDUCED
Heart muscle		91 $cm^{-1}mM^{-1}$ at 444 mμ
Yeast		87 $cm^{-1}mM^{-1}$ at 444 mμ
Bacillus subtilis		25 $cm^{-1}mM^{-1}$ at 444 mμ
	Acetobacter pasteurianum	120 $cm^{-1}mM^{-1}$ at 427 mμ

In tables 4 and 5 the values for heart muscle, yeast, and *Bacillus subtilis* are taken or calculated from the data of Chance (52). That for *Achromobacter fischeri* is unpublished data of Chance.

We can now sum up the established facts concerning bacterial cytochromes a, a_1, a_2 and a_3.

(a) A cytochrome of type a_3 so far has only been demonstrated definitely in *B. subtilis*, and this enzyme has a different substrate from the cytochrome a_3 of yeast and heart muscle. That is, the enzyme in *B. subtilis* is not cytochrome c oxidase.

(b) Cytochrome a occurs in some bacteria, even in one which does not contain cytochrome a_3. This will be discussed in the following section.

(c) The respiratory enzyme of *Acetobacter pasteurianum* has been defined as cytochrome a_1. This cytochrome or one with similar spectral properties also occurs in a number of other bacteria as part of a mixture of cytochromes that react with carbon monoxide. It seems likely that all pigments with α-bands at about 590 mμ are not identical with the enzyme of *Acetobacter pasteurianum*. Although *E. coli*, *P. vulgaris* and *Azotobacter chroococcum* show bands at this wavelength, the carbon monoxide inhibition of the respiration of these bacteria is only very slightly reversed by light.[6] Also, the data on the difference spectra (41) show that the relationship of the height of the Soret peak at 440 mμ to the visible peak at 590 mμ is different in a number of organisms that all show visible peaks at about 590 mμ.

The spectra of cytochromes a_1 and a_3 differ in the following respects: the α-band of cytochrome a_3 is around 605 mμ, whereas that of cytochrome a_1 seems to be at 590 mμ. The α-bands of the carbon monoxide compounds of both are at about 590 mμ; the γ-band of the cytochrome a_1-CO compound is at 427 mμ, while that of cytochrome a_3 is at 430 mμ. Thus, a very sensitive method is required to distinguish the two carbon monoxide compounds.

Tamiya and Tanaka (70) found that the rate of production of acetic acid by *Acetobacter pasteurianum* was unaffected by the oxygen concentration down to 2–3%. Thus, cytochrome a_1, like cytochrome a_3, has a high affinity for oxygen.

(d) Although cytochrome a_2 usually reacts with cyanide, carbon monoxide and oxygen, the bulk of evidence seems to argue against concluding that cytochrome a_2 is a bacterial cytochrome oxidase. The spectral characteristics of cytochrome a_2 are very different from those of

the other cytochromes. In the visible region it has a band in the oxidized form at about 647 mμ (47), and it seems to have little or no absorption in the Soret region. The suggestion, already discussed, that cytochrome a_2 has the properties of a biliviolin hemochromogen might explain the lability of this cytochrome; in such a structure, the iron is rather easily split off. On the other hand, there is some evidence that the cytochrome a_1 of *C. diphtheriae* (71) and of *E. coli* (72) has a heme nucleus similar to that of heart muscle cytochromes a and a_3, which is of the spirographis hemin type (73).

D. The New Type of Respiratory Enzyme

As discussed above, the pigment of *S. albus* which reacts with carbon monoxide gives a compound with a very different spectrum from the carbon monoxide compounds of cytochromes a_1 and a_3. The latter are similar to those of hemoproteins containing spirographis heme. The pigment of *S. albus*, on the other hand, has a prosthetic group more closely resembling protohemin. A critical and significant experiment concerning this cytochrome was done by Castor and Chance (74), who showed that it is the respiratory enzyme of these bacteria. The carbon monoxide action spectrum of *S. albus* was measured, and it was found to be the same as the photochemical dissociation spectrum and the carbon monoxide difference spectrum, with peaks at 567, 535 and 418 mμ. A trough is observed in the carbon monoxide difference spectrum at 430 mμ, indicating that the Soret peak of the reduced compound is in this region.

E. Relationship Between Cytochromes a and a_3

B. subtilis, which contains both cytochromes a and a_3 but in a different ratio from that in yeast, and *S. albus*, which has cytochrome a but no a_3, have proved to be good experimental material for the investigation of the relationship between these two cytochromes (75). The combination of cytochromes a and a_3 in heart muscle has resisted separation by physical means. We have made observations in the visible and Soret regions of the spectrum on the reactions of the pigments in these two kinds of bacteria with cyanide and carbon monoxide, using the sensitive spectrophotometric method designed by Chance (48).

Figure 11 illustrates the difference spectrum of the carbon monoxide compounds of the *B. subtilis* pigments. It can be seen that reduced pigments with peaks at 605 mμ and 445 mμ disappear (troughs in the difference spectrum) to form a carbon monoxide compound with peaks at 590, 540 and 430 mμ. These peaks are those corresponding to a cytochrome of type a_3, and the reduced cytochrome a_3 had peaks at 605 and 445 mμ. As has been mentioned, the ratio of the Soret to visible peaks of the carbon monoxide compound of *B. subtilis* was found by Chance (5) to be twice that of the compound in yeast.

However, the addition of carbon monoxide to anaerobic *S. albus* resulted in no change in spectrum in the region around 605 mμ (see figure

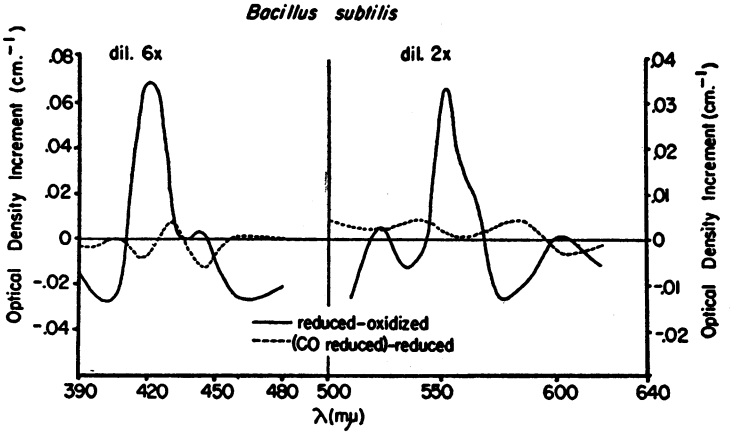

Figure 11. Difference spectrum of *Bacillus subtilis* (reduced-minus-oxidized) and carbon monoxide difference spectrum (carbon monoxide compound-minus-reduced).[5]

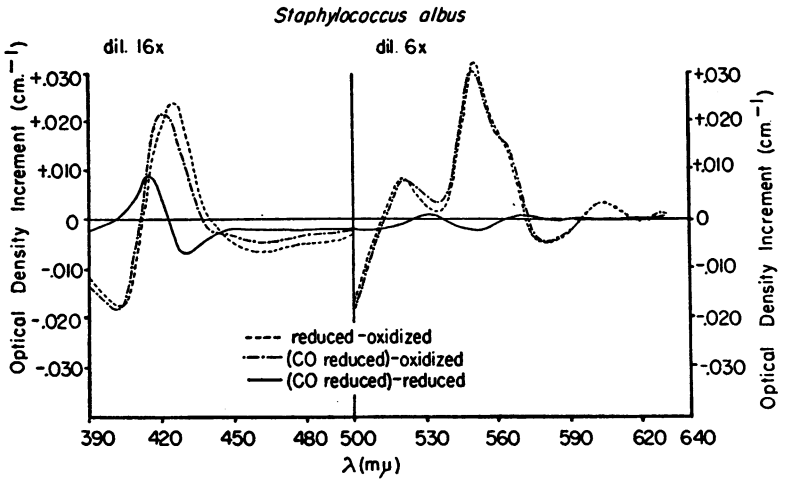

Figure 12. Difference spectrum of *Staphylococcus albus* (reduced-minus-oxidized) and carbon monoxide difference spectrum (carbon monoxide compound-minus-reduced).[5]

12), and the only compound formed was that of the respiratory enzyme described in Section III, D.

In the presence of cyanide there seemed to be a shift in the cytochrome a peak of *S. albus*, but it was found that the spectral changes following the addition of cyanide were due to its reaction with the catalase present in the bacteria and not to its reaction with the cytochrome a. In *B. subtilis* a similar reaction occurs between catalase and cyanide, and in addition, a trough in the difference spectrum at 445 mμ is observed, indicating that the cytochrome a_3 present had reacted also with cyanide.

These results with the bacterial pigments show that cytochrome a can exist separately from cytochrome a_3, and that the presence of a cytochrome a band cannot be taken as evidence for cytochrome a_3 in the cells. Our data disagree with the postulates that cytochromes a and a_3 are in reality one compound (76) or that a combination of three cytochrome a groups plus one cytochrome a_3 composes the system which reacts with oxygen in the cells (77). The data on the investigation of cytochrome a in the absence of cytochrome a_3 agree with the conclusion of Keilin that the former does not react with either carbon monoxide or cyanide.

All of the work on the reactions of the cytochrome systems emphasizes the importance of the formation of the carbon monoxide compound in establishing the identity of a given oxidase. Observations of spectral changes following the addition of cyanide may give misleading results because of the presence of catalase or peroxidase, which also react with cyanide. Catalase and peroxidase are not reduced by the reducing systems of the cells; thus, they do not react with carbon monoxide under physiological conditions since carbon monoxide reacts only with the reduced form of these enzymes.

F. Cytochromes b and b_1

The data from measurements of the difference spectra (table 1) show that some bacteria have peaks in the absorption spectra corresponding to what seem to be cytochromes b (564 mμ) and c (551 mμ); in other bacteria this pair is replaced by cytochrome b_1 (560 mμ). The combination of peaks at 552 and 560 mμ can also occur.

We found that the cytochrome b_1 peak of *P. vulgaris* is not due to the fused peaks of cytochromes b and c (see Section II, C). And Keilin (65), by observing the spectra at the temperature of liquid air, found that the cytochrome b_1 of *E. coli* was not a mixture of cytochromes b plus c. However, what appeared to be cytochrome b_1 in *B. subtilis* was actually a mixture of cytochromes b, c and e. Tissières (39) thought that the band of cytochrome b_1 in *Aerobacter aerogenes* seemed asymmetrical, with a reinforcement on the short wavelength side.

Pappenheimer made a study of what he called cytochrome b of *Corynebacterium diphtheriae*, which, from its spectral characteristics, seems

to be cytochrome b_1. Coulter and Stone (78) had demonstrated a relationship between toxin production by these bacteria and the excretion of a porphyrin into the medium. Then Pappenheimer and Hendee (37) showed that the relationship can depend upon the iron content of the medium, which also influences the content of cytochrome b_1 in the cells. The interesting hypothesis suggested, that diphtheria toxin might be the protein moiety of cytochrome b (79), has not been proven. Rawlinson and Hale (71) showed that the pyridine hemochromogen of the cytochrome b_1 of *C. diphtheriae* was identical with pyridine protoporphyrin IX; thus, it has the same prosthetic group as mammalian cytochrome b. However, the prophyrin excreted by *C. diphtheriae* into the medium is coproporphyrin III (80). This latter porphyrin is excreted by other bacteria (33), and it is present in some mycobacteria in such concentration that its spectral absorption bands can be seen (81).

Pappenheimer and Hendee (37) found that as the cytochrome b_1 content of the diphtheria bacteria increased, the succinoxidase activity of broken cell suspensions of the bacteria increased, and heat treatment or differential centrifugation resulted in no separation of the cytochrome b_1 and the succinoxidase activity. Comparison of the bacterial system with the heart muscle succinoxidase system showed that the two were different in a number of respects (82). The bacterial succinoxidase system was almost insensitive to 2.5×10^{-3} M cyanide and to some naphthoquinones, which are strongly inhibitory to the heart muscle system. Also the two systems showed different sensitivities to heat and pH. The bacterial cytochrome b_1, which they separated from the cells, had a different absorption spectrum from heart muscle cytochrome b, was more autooxidizable than the heart muscle enzyme, and could not replace the succinic dehydrogenase of heart muscle extract.

Although the cytochrome b_1 content of the extracts of *C. diphtheriae* seemed to parallel the succinic dehydrogenase activity, it cannot be considered proven that cytochrome b_1 is succinic dehydrogenase. In heart muscle, there is abundant evidence that cytochrome b is not identical with succinic dehydrogenase (83), and the kinetic data of Chance (45) throw doubt upon the participation of cytochrome b in the main pathway of electron transfer from succinate to oxygen by the heart muscle succinoxidase system.

Other suggestions have been made that iron enzymes or other heavy metal catalysts may be involved at the dehydrogenase level in bacteria. In *Aerobacter aerogenes* grown in a metal-deficient medium, the decreased rate of oxygen uptake when succinate or lactate was oxidized could be correlated with a decrease in succinic dehydrogenase or lactic dehydrogenase activities (39). The rate of oxygen uptake in the presence of glucose was unchanged, and so was the rate of reduction of methylene blue on addition of glucose. Our own experiments with *Aerobacter aerogenes* showed that the rate of reduction of the cytochromes in the presence of succinate was markedly decreased in the presence of carbon monoxide. These observations are reminiscent of the finding of Gilder and Granick (84) that a strain of *Hemophilus influenzae* required heme for anaerobic as well as for aerobic growth. Also, Bach *et al.* (85) having purified cytochrome b_2 from yeast, found that it seemed to be related to the activity of the yeast lactic dehydrogenase.

Two other reactions of cytochrome b_1 have been postulated. The first is that this cytochrome is related to the "nitrate reductase" activity of bacteria. The work of Granick and Gilder (86) and of Quastel (87) had indicated that an iron-porphyrin enzyme is involved in this activity. Then Sato and Egami (88) observed that the intensity of the nitrate reductase activity in crude preparations from *E. coli* roughly paralleled the strength of the cytochrome b_1 band (which they refer to as "bacterial cytochrome b"). Also, if the cytochrome b_1 of the preparation was reduced by the formic dehydrogenase system, cytochrome b_1 was partially oxidized by the addition of nitrate. Their work shows only that cytochrome b_1 can react somewhere in the pathway of electron transport to the nitrate reductase system. Joklik (89) has made further purification of the enzyme but did not report any relationship to cytochrome b_1.

Eddy *et al.* (90) postulated that the reduction of dehydroascorbic acid by certain bacteria, notably staphylococci and coliforms, might be related to cytochrome b_1. Our difference spectra showed, however, no evidence of cytochrome b_1 in three strains of staphylococci.

Altogether, there is no definite evidence concerning the roles of cytochromes b or b_1 in bacteria. In yeast, at least part of the band cor-

responding to what has been known as cytochrome b can be attributed to the H_2O_2-complex of the peroxidase present (91). Similar experiments have not been made with bacteria.

Egami *et al.* (92) extracted and partially purified a soluble cytochrome from some halotolerant bacteria. The reduced pigment showed peaks in the absorption spectrum at 554, 521 and 415 mμ. Although they concluded that the hemin part of the pigment is different from that of either cytochrome b or cytochrome c, they have called the pigment cytochrome b_4. The physiological activities of the pigment were not investigated.

G. Cytochrome c

Many workers have concluded that cytochrome c was present in the bacteria they examined spectroscopically although there is disagreement among the various reports (13, 14, 15, 56). The difference spectra of a number of bacteria possess a peak at 552 mμ. However, the presence of an absorption peak at about 550 mμ is only presumptive evidence for the presence of cytochrome c; the isolation and identification of the pigment from the cells are necessary to establish its presence there. Since most bacterial oxidases will not oxidize mammalian cytochrome c at an appreciable rate, and the cytochrome with a peak at about 550 mμ in bacteria can be seen to be oxidized rapidly in the intact cells, it seems unlikely that the pigment observed is identical with mammalian cytochrome c.

In *E. coli commune*, Keilin and Harpley (65) demonstrated that cytochrome c was not present, and Pappenheimer (93) could find no evidence for it in cells of *Corynebacterium diphtheriae*. Militzer *et al.* (94) saw spectral bands corresponding to what they thought was reduced cytochrome c in a strain of thermophilic bacteria, but attempts to isolate it from the cells led to the conclusion that the pigment was different from cytochrome c. The substance they obtained was rather insoluble, was not removed from the particles by precipitation with acid, and could not be extracted with trichloracetic acid. Also the pigment isolated was not active in the beef heart cytochrome oxidase system.

Now it has been shown that several pigments exist with absorption spectra similar to that of heart muscle cytochrome c, but which differ from it in a number of other properties. Neilands (95) isolated a pigment from the smut fungus *Ustilago sphaerogena* that has an absorption spectrum identical with that of heart muscle cytochrome c, but the protein part differed. There seems to be a group of compounds with the same prosthetic group as cytochrome c, but with different protein moieties. Tint and Reiss (96) even found differences in the isoelectric points of cytochrome c from the heart muscle of different animal species. The cytochrome c from the ustilago was reported to be active in the rat liver succinic oxidase system.

Pigments with spectra similar to that of cytochrome c have been isolated from *Rhodospirillum rubrum* by Vernon (97), from *Pseudomonas fluorescens* by Lenhoff and Kaplan (35), and from *Azotobacter vinelandii* by Wilson and Wilson,[7] all of which are reported to be enzymatically different from heart muscle cytochrome c. Elsden, Kamen and Vernon (97a) found that the rhodospirillum pigment which is a soluble cytochrome is not oxidized by mammalian cytochrome oxidase and suggest the name cytochrome c_2 for it. The cytochrome from the pseudomonad was not reduced by TPN-cytochrome c reductase of liver but was oxidized on addition of an extract of the bacteria, while mammalian cytochrome c was not. The only other cytochromes that have been isolated from bacteria are that from the halotolerant organisms (see Section III, F) and that from *Chlorobium limicola* (Section II, A). These cytochromes also show spectra that are similar to but not identical with that of cytochrome c. It is not known whether there are any similarities in the activities of these various pigments.

H. Action of Antimycin A

In the chain of oxidative enzymes in mammalian tissues, some evidence exists for the presence of still one more link in addition to the oxidase, the other cytochromes and the dehydrogenases. Substances such as BAL and antimycin A inhibit the reactions of the whole oxidative system, seeming to act at a point somewhere between the dehydrogenases and cytochrome c (98, 99), so that cytochromes a and c are not reduced, and cytochrome b cannot be oxidized. We have tested the effect of antimycin A on the respiration and the difference spectra of the following bacteria: *Acetobacter pasteurianum*,

[7] Wilson, T. G. G., and Wilson, P. W., unpublished experiments.

Aerobacter aerogenes, *Azotobacter chroococcum*, *Bacillus subtilis*, *Escherichia coli*, *Micrococcus lysodeikticus*, and *Staphylococcus albus* (41). No effect has been observed with any of these microorganisms, but the possibility has not been eliminated that the antimycin does not penetrate the cells or that it is bound by other substances present (100). However, it does react in some manner to disrupt the chain of oxidation reactions in yeast cells. Thus, one more difference between the bacterial systems and that of mammalian tissues is apparent.

I. Association of the Bacterial Cytochrome Systems with Insoluble Cellular Material

As in mammalian tissues, the cytochromes of bacteria seem to be associated with insoluble material within the cell. Moyed and O'Kane (101) separated from *P. vulgaris* by sedimentation at 20,000 g a fraction which contained the cytochrome system of these organisms. The cytochromes could be reduced by a soluble pyruvate dehydrogenase which they purified from the supernatant fluid, and a mixture of the purified dehydrogenase plus the insoluble fraction formed an active system that linked the dehydrogenase through the cytochrome system to molecular oxygen. Stanier *et al.* (102) examined fractions obtained from *Pseudomonas fluorescens* broken by exposure to sonic oscillations. They separated coarse particles (10–100 mμ in diameter), fine particles (less than 1 mμ in diameter), and a soluble fraction. Both the coarse and the fine particles showed the same enzymatic activities and contained several dehydrogenases and the cytochrome system of the bacteria; thus, the oxidation of a number of substrates by oxygen was observed. Examination of repeatedly washed suspensions of coarse particles by means of the electron microscope revealed that the material was physically heterogeneous.

Observations of Wilson and Wilson[7] with sonic extracts of *Azotobacter vinelandii* and in our laboratory[8] with *Acetobacter suboxydans* and *Staphylococcus albus* broken in a number of different ways also show that a whole range of sizes of insoluble "particles" is obtained from the extracts, all of which have similar enzymatic activities and apparently differ only in size. The particles of the azotobacter contain the bacterial cytochrome system and malic, lactic and succinic dehydrogenases. The insoluble material from *Acetobacter suboxydans* contains the complete systems that oxidize alcohol, glucose and lactate, and the rapid oxidation and reduction of the cytochromes can be observed as in the whole cells. Thus, the particulate material is useful for examining the nature of these respiratory enzymes. No separation of the cytochromes or of the enzymatic activities of the *Acetobacter suboxydans* particles was observed when the insoluble material was separated into fractions by differential centrifugation from 10,000 to 110,000 g. In the extracts of both *Azotobacter vinelandii* and *Acetobacter suboxydans*, a small fraction of the particulate material resists sedimentation by a high centrifugal force (about 140,000 g for 60 minutes for the azotobacter and 110,000 g for 30 minutes with the acetobacter). The light-scattering properties of these fractions are low, so that measurements of absorption spectra can be made in an instrument such as a Beckman spectrophotometer.

Weibull (103, 104) was able to digest the cell walls of *Bacillus megaterium* by treatment with lysozyme in sucrose solution and to isolate the bacterial protoplasts. The protoplasts then were lysed by treatment with buffer. Phase contrast microscopy of a fraction of the lysate revealed "ghosts", which Weibull's data indicate must consist of cytoplasmic material and may represent the plasma membrane. The "ghost" fraction was observed to contain the entire cytochrome system of the bacteria. The ghosts were disrupted by treatment with sonic oscillation to form an insoluble fraction that could be sedimented as a uniformly colored pellet in the preparative ultracentrifuge.

Militzer *et al.* (94, 105) also obtained an insoluble fraction by digestion of a thermophilic organism, *Bacillus stearothermophilus*, with lysozyme. The insoluble fraction was red in color and contained malic and succinic dehydrogenases and the bacterial cytochrome system. Georgi *et al.* (106) and Burns and Militzer (107) described the "red fraction" as made of spherical bodies whose volume is approximately that of the cells from which they are derived. As has been suggested by Stanier *et al.* (102) and by Weibull (104), the red fraction may consist of "ghosts" containing other cytoplasmic material.

It appears that all of the observations on cytochrome bearing fractions obtained from bacteria

[8] Smith, L., and Kuby, S. A., unpublished experiments.

agree best with the postulate of Weibull that these enzymes are associated in the cell with a structure such as the cytoplasmic membrane or a similar structure of uniform nature. The wide range of sizes of "particles" obtained by the usual methods of breaking bacteria would then result from the disruption of this structure. A knowledge of the nature of the insoluble material will be important in attempts to separate and purify the bacterial cytochromes.

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